

Choosing preclinical study models of diabetic retinopathy: key problems for consideration

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Abstract: Diabetic retinopathy (DR) is the most common complication of diabetes mellitus in the eye. Although the clinical treatment for DR has already developed to a relative high level, there are still many urgent problems that need to be investigated in clinical and basic science. Currently, many in vivo animal models and in vitro culture systems have been applied to solve these problems. Many approaches have also been used to establish different DR models. However, till now, there has not been a single study model that can clearly and exactly mimic the developmental process of the human DR. Choosing the suitable model is important, not only for achieving our research goals smoothly, but also, to better match with different experimental proposals in the study. In this review, key problems for consideration in choosing study models of DR are discussed. These problems relate to clinical relevance, different approaches for establishing models, and choice of different species of animals as well as of the specific in vitro culture systems. Attending to these considerations will deepen the understanding on current study models and optimize the experimental design for the final goal of preventing DR.

Keywords: animal model, in vitro culture, ex vivo culture, neurovascular dysfunction

Introduction

The worldwide incidence of diabetes is rising quickly. Studies have shown that after 10 years of diabetes, nearly 50% of type 2 and 70% of type 1 diabetic patients will develop diabetic retinopathy (DR).¹ As a leading cause of blindness in eye diseases, DR drives much attention, on the part of clinical and basic researchers, to the investigation of its mechanism and prevention. However, the pathogenesis of DR is complicated, so validated study models are needed to deepen our understanding of the disease mechanisms and for trying new approaches for early screening and therapeutic intervention.

DR is a common clinical condition. As to study models for DR, there are several different species of animal models, from tiny zebrafish to monkeys, and in vitro culture systems for retinal blood vessel assay, using human or nonhuman tissues. All of these models are aimed at mimicking the clinical course of DR, while each has its limitation in matching the full features of the human disease. Previously, there have been some reviews describing animal models of DR in great detail.²⁻⁶ In addition, there are other reviews focusing on angiogenesis models in vivo⁷ and in vitro.⁸ However, when a researcher is faced with choosing the study model for different purposes, there are many key problems to consider in advance.

In this review, a summary of the most common and important problems for consideration on the selection of appropriate study models of DR is provided. The features and challenges of clinical DR that should be considered in preclinical studies are discussed first. Then, the current research focuses in clinical and basic science are

summarized, for researchers to consider and compare with their own experimental goals. Finally, the popular modeling approaches in common animal species, together with their advantages and limitations, are summarized. These considerations will help researchers to select the appropriate candidate when facing multiple choices of study models.

Clinical considerations

Characteristics of human DR

Clinically, DR is mainly classified in two groups, nonproliferative DR (NPDR) and proliferative DR (PDR). PDR is judged by the presence of retinal neovascularization,⁹ which is usually confirmed with fluorescence fundus angiography imaging. PDR is the more advanced stage of DR. In PDR, proliferating neovasculature contributes to severe complications, eg, vitreous hemorrhage, retinal scars, and tractional retinal detachment, all of which often need vitreoretinal surgery. However, the endpoint of PDR is variable, and irreversible vision loss is attributed to retinal structure damage and layer thinning. Thus, the Early Treatment of Diabetes Retinopathy Study (ETDRS) has staged NPDR with mild, moderate, and severe grades, aimed to screen risk factors and to detect the earlier stages of DR for nonsurgical treatment, eg, retinal photocoagulation or antiangiogenic therapy.^{10,11}

Histologically, retinal vascular lesions are considered to be the hallmark and the grading criterion of DR. The first visible alteration in retinal vasculature is the formation of microaneurysms. The further changes are intraretinal focal hemorrhage, venous beading, and intraretinal microvascular abnormalities (IRMAs) showing with microvascular torsion and regional capillary nonperfusion on fluorescence fundus angiography imaging. IRMA is associated with “cotton wool” spots observed with funduscopy, which are focal infarcts of nerve fibers in essence. Following the progression of vascular damage, diabetic macular edema appears, which is one of the major causes of vision loss in DR, linking with lesions at the blood–retinal barrier.^{12,13}

Clinical challenges

A current clinical challenge for consideration in diagnosis and prevention is the screening for risk factors that contribute to retinal vascular abnormality, for eg, studies of ocular blood flow;^{14,15} biomarkers detected in body fluids, like advanced glycation end products (AGEs); interleukins; and tumor necrosis factor- α (TNF- α).^{16,17}

As to nonsurgical therapeutic investigations, the focus has been on treating diabetic macular edema and

antiangiogenesis, and on vascular cell protection to inhibit neovascularization, for eg, with vascular endothelial growth factor (VEGF) inhibitor¹⁸ or other potential ocular-delivered drugs.^{19–22} Recently, anti-VEGF has become one of most important discoveries to treat neovascularization clinically. However, limitations to anti-VEGF therapies also exist, eg, resistance, systemic side effects with local use, and non-VEGF-related vascular proliferative mechanisms.^{18–22} Indeed, other than VEGF, more and more potentially angiogenic factors have been discovered, such as insulin-like growth factor 1 (IGF-1),²³ platelet-derived growth factor B (PDGF-B), erythropoietin (EPO), angiopoietin 2,²⁴ interleukin 8 (IL-8), etc.²⁵ Antioxidants, eg, alpha-lipoic acid (ALA), are other potential drugs that prevent micro- and macrovascular damage, by normalizing pathways downstream of mitochondrial overproduction of reactive oxygen species, and preserve pericyte coverage of retinal capillaries.^{26,27}

As to surgical aspects, treatment advances are largely dependent on development of new instruments for the goal of perfecting results of vitreoretinal surgery. However, when DR is advanced to the proliferative stage, surgical effect is limited to the severe damage of retinal neurovascular structure.^{28,29}

Thus, more and more preclinical trials need to use different *in vivo* animal models or *in vitro* vascular assays to understand the mechanisms and to find potential treatment to prevent human DR.

Choices of study model in DR

Principle of choice: Research goal is the basic consideration

Understanding of the mechanism of DR is complicated by the complexity of the retinal structure. The retina contains multiple cell components and structure, and when exposed to the stress of hyperglycemia, not only blood vessels but also, retinal neurons and glial cells will all be challenged by the insult. The concept of the neurovascular unit is a kind of simplified understanding of the crosstalk between the retinal blood vessel and other cells around it.³⁰ From the view of a neurovascular hypothesis in DR, conditions of hyperglycemia induce vascular damage, thereafter destroying the microenvironment and the crosstalk between endothelial cells/pericytes and neurons, endothelial cells/pericytes and retinal glial cells, as well as neurons and glial cells, finally leading to vascular proliferation, glial proliferation, and neural degeneration (Figure 1).

Previously, the focus was on establishing various animal models of DR, and there were many classic studies

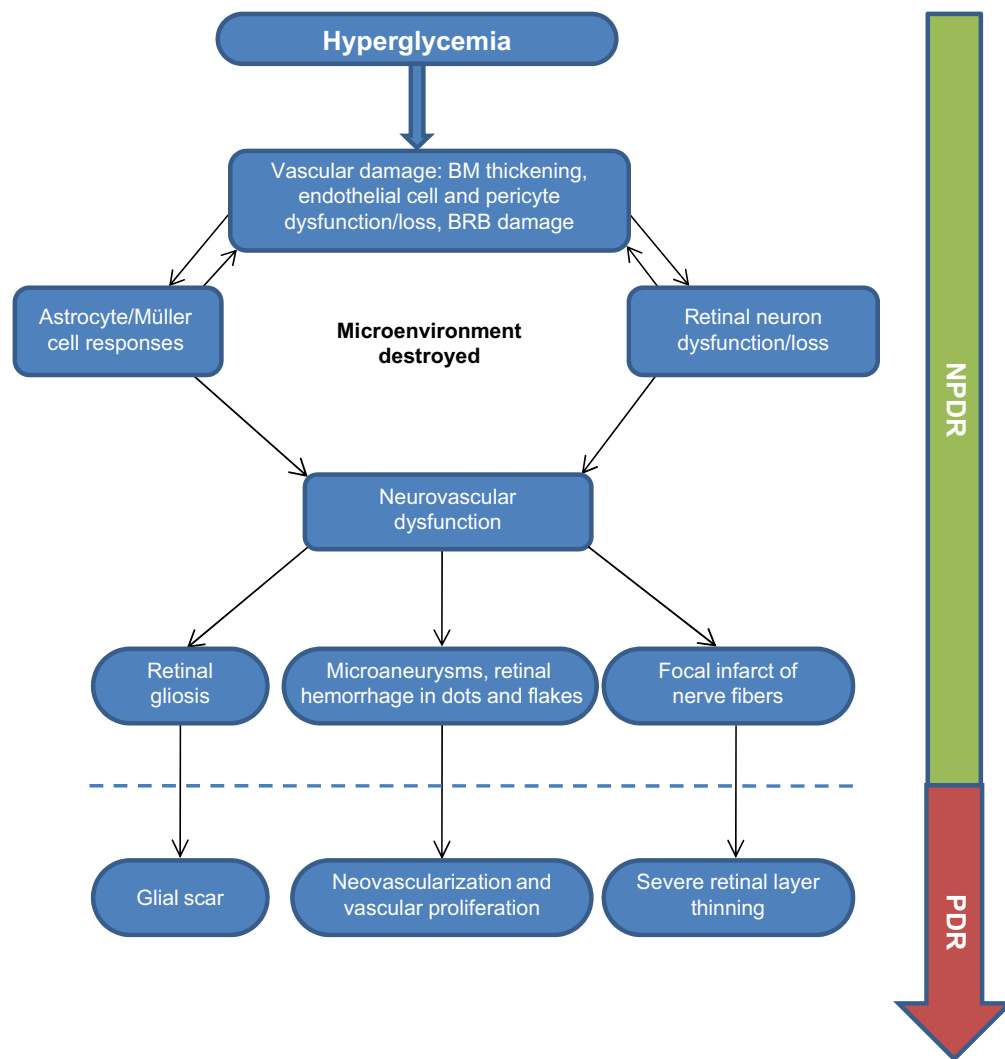


Figure 1 Neurovascular hypothesis for the pathogenesis of diabetic retinopathy.

Abbreviations: BM, basement membrane; BRB, blood–retinal barrier; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

investigating morphological damage to the retinal vasculature and comparing this to clinical features found in human DR. Studies have shown that one of the earliest histological changes of DR is thickening of the capillary basement membrane.³¹ Dysfunction of endothelial cells and pericytes, and thereafter cell loss, are also early phenomena, which could be responsible for the acellular capillary formation.⁴ It is believed that the formation of microaneurysms, the hallmark alteration of human DR, are attributed to the loss of pericytes.³² Cotton wool spots are linked with focal IRMAs.³³

Therefore, we summarized the current research areas and categorized them with respect to the different stages of development of DR and according to their different goals. At different stages of NPDR and PDR, there have been different points of focus, for eg, in NPDR, the blood vessel-related research focuses on early biomarkers to detect vascular

dysfunction,³⁴ the mechanisms for how hyperglycemia induces vascular damage,³⁵ and potential treatment to protect endothelial cells or pericytes from dysfunction or loss.²² Neuron-related research has focused on early detection of neural degeneration by electrophysiological methods,^{36,37} and neuroprotection.^{38,39} Glial cell-related research focuses on the response of retinal astrocytes and Müller cells.³⁹ Microenvironment damage-related research directions include cytokines and the mechanisms of inflammation, as well as immune-associated mechanisms, etc.⁴⁰ As to preventing PDR, the current research goals relate to potential therapies for inhibiting or treating the complications of neovascularization, eg, anti-VEGF, which was mentioned above. Thus, after clarifying the goal of investigation and the related stage in DR, researchers can pay attention to the choices for different approaches and study models.

Different choices to establish study model of DR

Currently, in the establishment of animal models for DR, there are mainly three approaches: 1) inducing experimental hyperglycemia, 2) using animals with spontaneous diabetes mellitus, and 3) inducing retinal angiogenesis without diabetes mellitus.

As to the approach of experimental hyperglycemia induction, this mainly includes: a) pharmacological methods, for eg, intraperitoneal injection of streptozotocin (STZ)^{41–45} or alloxan;⁴⁶ b) diet methods, such as feeding a high-galactose diet;⁴⁶ and c) surgical method, for eg, Pancreatectomy.⁴⁷

a) Regarding induction of pharmacological hyperglycemia, intraperitoneal injection using STZ is the classic and most popular approach to establish diabetic animal models. The mechanism is that STZ can kill pancreatic β cells by its toxicity. Following the hyperglycemia, the animal will develop DR. Compared with the other methods listed above, STZ injection is time-saving and efficient and has been used widely in rodents and large animals, for eg, rabbits, cats, dogs, pigs, and monkeys. The standard modeling protocol that has been established in mice was suggested by the Animal Models of Diabetic Complications Consortium.⁴⁸ It has been reported that the STZ-induced model could demonstrate NPDR, however, rarely forming microaneurysms.⁴⁹ In addition, only a few studies reported the formation of focal neovascularization.⁵⁰ Alloxan is another drug used to induce hyperglycemia. However, compared with STZ, the alloxan-induced model has needed longer time to demonstrate a milder level of pathological change of DR – reportedly 3 months in mice⁴⁶ and 12 months in rats.⁵¹

b) The high galactose-diet feeding method can be used in rodents and other large animals. Compared with the STZ method, it is time-consuming. However, it is suitable for zebrafish, by just directly elevating the concentration of glucose in the fishpond.⁵²

c) Surgical methods, like pancreatectomy, can be used in large animals, such as cats,⁴⁷ pigs,⁵³ and monkeys.⁵⁴ However, it is also time-consuming, for eg, in one report using cats, although hyperglycemia was induced quickly, within 1–2 weeks after the surgery, DR and formation of microaneurysms was observed only after around 5 years.⁴⁷ Generally, experimental hyperglycemia-induced methods can be applied in wide types of animals, without obvious species limitation.

As to the approach of using spontaneously diabetic animals for study of DR, this mainly refers to use of genetic technology

to create animals that develop hyperglycemia and diabetes mellitus spontaneously, followed by the development of the pathological changes of DR. The spontaneous DR model has been established using mice, rats, and zebrafish. The DR animal created under this method shares the advantage of consistent phenotypes and high successful rate of induction. However, higher cost and genetic technology are needed. In addition, compared with experimentally induced-DR methods, spontaneous diabetic animals need a longer period to demonstrate DR. There are a relatively small number of animals with special strains that can develop diabetes spontaneously, but this has been reported in rats and monkeys (Table 2). Similarly, these animals with intraspecies variation also need a longer period to demonstrate DR, while the phenotypic variation is larger than for genetically mutated animals.

As to the method of retinal angiogenesis-induction, this refers to inducing retinal blood vessel proliferation without elevation of the blood glucose. The popular methods include oxygen-induced retinopathy and VEGF ocular delivery. Currently, the oxygen-induced retinopathy method is standardized and popularly applied in mice⁵⁵ and rats,⁵⁶ while the method of VEGF ocular delivery is more suitable for larger animals, for eg, rabbit and monkey.⁵⁷ Technically, these are not specific DR models, since they share phenotypes with other retinal neovascular diseases, such as retinopathy of prematurity, retinal vein occlusion, and retinal artery occlusion. However, from the view of pathogenesis, neovascularization is the same key pathogenesis in PDR. In addition, the angiogenic method can induce advanced vascular proliferation to mimic PDR, while the hyperglycemia-induced models mostly mimic NPDR. We summarized the rationale, advantages, and limitations for each popular modeling approach currently used in DR in Table 1.

Animal choices in vivo and cell culture choices in vitro

Genetically, mouse and zebrafish have the greatest potential as in vivo animal models of DR for the investigation of mechanisms and preclinical drug screening. They have advantages such as similar gene background to humans and ease of manipulation by the current genetic technology. In addition, the small size of animal is also easy to handle and house, and the short lifespan and large breeding rate can shorten the experimental period. These types of animals are inexpensive themselves, although gene modification enhances their cost. However, with the maturation of gene technology, more and more commercial gene-modified animals have already been made available for purchase for

Table 1 Comparison of popular methods for modeling of different animals of DR

| Animal | NPDR | | PDR |
|--|--|-----------------------------------|--|
| Mouse | 1) STZ injection ⁴¹⁻⁴⁵ 2) Alloxan injection ⁴⁶ | Galactose-fed ⁴⁶ | Transgenic/Gene mutated mice: Akita mice, ⁵⁸ NOD mice, ⁷² db/db mice, ⁵⁹ KKAY mice ⁶⁰ OIR ⁵⁵ |
| Rat | 1) STZ injection ⁷⁵ 2) Alloxan injection ⁵¹ | Galactose-fed ⁷⁰ | Gene/Substrain modified rats: BB rats, ⁷³ ZDF rats, ⁷⁶ OLETF rats, ⁷⁷ GK rats, ⁷⁸ SDT rats ⁷⁹ OIR ⁵⁶ |
| Large animals (rabbits, cats, dogs, pigs, monkeys) | 1) STZ injection: rabbits, ⁸¹ dogs, ⁸² pigs, ⁸³ monkeys ⁵⁴ 2) Alloxan injection: pigs ⁸⁴ | Galactose-fed: dogs ⁷¹ | Others: 1) VEGF intravitreal delivery: monkeys ⁵⁷ 2) Pancreatectomy: cats, ⁴⁷ pigs, ⁵³ monkeys ⁵⁴ 3) Spontaneous obese monkeys ⁸⁵ VEGF intravitreal delivery: rabbits ⁵⁷ |
| Zebrafish | STZ injection ⁶³ | Glucose-fed ⁶² | Transgenic/Gene mutation: <i>wh1^{-/-}</i> zebrafish ⁶² HIR ⁶⁴ |
| In vitro culture | | | In vitro culture of endothelial cells ⁸⁶ Ex vivo retinal culture ^{87,88} |

Abbreviations: DR, diabetic retinopathy; GK, Goto-Kakizaki; HIR, hypoxia-induced retinopathy; NOD, nonobese diabetic; NPDR, nonproliferative diabetic retinopathy; OIR, oxygen-induced retinopathy; OLETF, Otsuka Long-Evans Tokushima fatty; PDR, proliferative diabetic retinopathy; SDT, spontaneously diabetic Torii; STZ, streptozotocin; VEGF, vascular endothelial growth factor; WBN/Kob, Wistar Bonn/Kobori; ZDF, Zucker diabetic fatty.

Table 2 Comparison of rationale, advantages, and limitations of different modeling approaches of DR

| Approach | Rationale | Advantages | Limitations |
|--|--|---|--|
| STZ injection ⁴¹⁻⁴⁵ | Destroys pancreas, leading to hyperglycemia | 1) Classic method 2) Fast induction | 1) Need to monitor the blood glucose level to confirm the presence of hyperglycemia 2) Mostly forms NPDR, rarely forms PDR |
| Galactose-fed ^{46,70,71} | Induces hyperglycemia through diet | Applicable to zebrafish, when galactose replaced by glucose in fishpond | 1) Need longer period to onset of retinopathy in mammals 2) Mostly forms NPDR, rarely forms PDR |
| Pancreatectomy ^{47,53,54} | Removes pancreas to lose the control of blood glucose by insulin | Applicable to large animals, like cat and monkey | 1) Large variation among individuals and species 2) Need longer period to onset of retinopathy 3) Mostly forms NPDR, rarely forms PDR |
| Transgene/Gene mutation ^{58,62,72,73} | Effect on the spontaneous degeneration of pancreatic β cells | 1) Consistent phenotype 2) High success rate of hyperglycemia | 1) High cost 2) Needs genetic technology 3) Mostly forms NPDR, rarely forms PDR; only Akimba transgenic mice were reported to show PDR formation ⁶¹ |
| OIR ^{55,56} | Angiogenesis and neovascularization | 1) Definite neovascularization | 1) Non-DR-specific 2) Applicable to newborn rodents |
| Ocular delivery of VEGF ⁵⁷ | VEGF-induced angiogenesis | 2) Classic method Theoretically induce neovascularization directly | 1) Non-DR-specific 2) Large variation among individuals and species 3) After metabolized, effects of VEGF disappear 4) Suitable for large animals ⁵⁷ |

Abbreviations: DR, diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; OIR, oxygen-induced retinopathy; PDR, proliferative diabetic retinopathy; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

achieving different experimental goals with a not very high price. Detailed description of the characteristics of these genetic animals is beyond the purpose in this review; we listed the related references in Table 2. In addition, double-gene transgenic or fluorescent gene-added animals extend our understanding and deepen our investigation of the molecular nature of human diseases. These are unique advantages when compared with other animal models.^{58–62}

Experimentally, mice and rats are still the most popular models of DR, not only because of the advantages mentioned above, but also since there have been many standardized protocols of modeling approaches available for reference to researchers, such as the STZ-injection model, oxygen-induced retinopathy model, and genetic model. In addition, rodents are suitable for the vast majority of detection reagents used in the laboratory. Monkey models share the most similar features of human DR compared with other animal models, which could be applied in research fields like live imaging examinations, diagnosing biomarker screening, and medical or surgical therapeutic trials; however, the approaches for monkeys are far from mature when compared with those for rodents. In addition, the large size, long lifespan, and inevitable ethical requirements have largely limited their applications. Other large animals, like cats and dogs, have large-sized eye balls, which are more convenient for handling than rodent eyes. However, their gene backgrounds are far from human, the number of suitable experimental reagents is far less than for rodents, and their large size needs more housing space, which all limited their usage as the choice of animal models for DR. However, compared with all the above species of animals, the zebrafish has its own advantages as a potential DR model, for eg, the experimental methods of STZ injection,⁶³ high-glucose diet feeding,⁵² and hypoxia-induced retinopathy (HIR)⁶⁴ are all convenient to perform in zebrafish, and the zebrafish with the gene *vhl*^{-/-} mutation is a spontaneous VEGF-overexpressing animal model, which makes it suitable to mimic the development of PDR⁶². However, the less similar retinal vascular structure to human, difficult handling, because of tiny size, and lack of reagents for molecular studies still limit its usage.

Compared with the *in vivo* animal model, *in vitro* culture systems can be used as experimental models of retinal angiogenesis. Since pathological angiogenesis of the retina is the key cause of progression in PDR, the retinal angiogenesis assay is suitable for screening novel antiangiogenic drugs before testing *in vivo* animals and in patients clinically.

In this area, study about various VEGF blockers is the most important direction in recent years. Several generations have been updated, and some of them have been applied clinically to patients, for eg, pegaptanib, ranibizumab, aflibercept, and bevacizumab.^{65,66}

Currently, there are two main kinds of *in vitro* culture proposals. Traditional *in vitro* cell culture uses isolated endothelial cells or pericytes, in particular, to maximally mimic the human angiogenic process; human-source endothelial cells and pericytes can be used more conveniently than *in vivo* study. Moreover, retina-derived endothelial cells can be applied⁶⁷ for the search of specific inhibitors of retinal neovascularization. However, the traditional *in vitro* culture has its weakness since without the microenvironment of retina, isolated cells may possibly lose their responses to angiogenic molecules. Thus, another culture proposal has been created, which is the *ex vivo* retinal model. The *ex vivo* model is established with retinal culture. In PDR, isolating the retina from the *in vivo* DR model for culture *in vitro* can be useful for studying the role of proangiogenic factors or antiangiogenic factors in the development of retinal neovascularization.^{23,25,68,69} We summarized the popular methods applied in common modeling animals and culture systems in Table 2.

Conclusion

The experimental model is the link between basic and clinical research. It deepens our understanding of the mechanisms and supports us in discovering novel therapeutic interventions to prevent the progression of DR. However, each DR model has its own advantages and limitations. Careful consideration in choosing the model helps us achieve the research goal with high efficiency. The preclinical modeling experiments are ultimately beneficial to clinical patients.

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Disclosure

The authors report no conflicts of interest in this work.

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